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Transcription Factor Control in Neuronal Maintenance and Survival

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Stockholm 2010

Cover

Back cover:
Confocal microscopy image showing the dopamine neurons in the midbrain of a transgenic mouse line. The image demonstrate the expression of Tyrosine hydroxylase in green, β-Galactosidase in red and Cre recombinase in blue as assessed by triple fluorescence immunostaining.

Front cover:
A colour-inverted picture of the back cover image.

Published by Karolinska Institutet. Printed by Larserics Digital Prints AB.

ISBN 978-91-7409-897-6

ABSTRACT

The Transcription factor (TF) Nurr1 is essential for the development of a group of dopamine neurons that are located in the ventral midbrain (also referred to as mesencephalon). A progressive pathology of these midbrain dopamine (mDA) neurons is evident in Parkinson's disease, the most frequent neurodegenerative movement disorder. In addition Nurr1 is expressed in a number of other CNS areas. In the hippocampus, Nurr1 is co-expressed with its homologues Nur77 and Nor1, collectively named for NR4A TF's. These TF's are rapidly and strongly induced in response to stressful stimuli and an acute *NR4A* induction has been noted in e. g. hippocampal, cortical and striatal neurons after ischemia, seizures and focal brain injury in rodents. However, the functional roles of the stress-induced NR4A-expression have remained unknown. As Nurr1 is critical for the mDA neuron development and is widely expressed in the adult CNS, we hypothesized that Nurr1 might also play a crucial role in maintaining mature neurons. To address this hypothesis, the functional consequences of gain-of-function or loss-of-function of NR4A proteins were assessed in maturing and adult neurons *in vitro* and *in vivo*. The research presented in **paper I & II** describes the consequences of spatiotemporal ablation of *Nurr1* in mDA neurons in mice. Our results revealed that Nurr1 continues to be critical for the maturing mDA neurons and for maintaining a DA phenotype in the mDA neurons of adult mice. In **paper III**, the function of stress-induced NR4A proteins was characterized *in vitro* in cultured neurons and *in vivo* in the hippocampi of mice. The data revealed that stress-induced NR4A promoted neuroprotection in neurons, presumably by up-regulating a subset of neuroprotective genes. The work in **paper IV** deals with the mechanism by which Nurr1 mediates transcriptional activation. We identified a novel putative Nurr1 coregulator-binding site that might recruit as yet unknown coregulators.

LIST OF PUBLICATIONS

I. **Banafsheh Kadkhodaei**, Takehito Ito, Eliza Joodmardi, Bengt Mattsson, Claude Rouillard, Manolo Carta, Shin-Ichi Muramatsu, Chiho Sumi-Ichinose, Takahide Nomura, Daniel Metzger, Pierre Chambon, Eva Lindqvist, Nils-Göran Larsson, Lars Olson, Anders Björklund, Hiroshi Ichinose and Thomas Perlmann (2009). Nurr1 is required for maintenance of maturing and adult midbrain dopamine neurons. Journal of Neuroscience 29 (50): 15923-32.

II. **Banafsheh Kadkhodaei** and Thomas Perlmann (2010). Maintenance of Midbrain Dopaminergic Neurons. Manuscript.

III. Nikolaos Volakakis, **Banafsheh Kadkhodaei**, Eliza Joodmardi, Karin Wallis, Björn Vennström, Bruce M. Spiegelman and Thomas Perlmann (2010). NR4A Orphan Nuclear Receptors Trigger a Neuroprotective Pathway Induced by Elevated Cyclic AMP. Manuscript.

IV. Nikolaos Volakakis, Michal Malewicz, **Banafsheh Kadkhodai**, Thomas Perlmann and Gerard Benoit (2006). Characterization of the Nurr1 ligand-binding domain co-activator interaction surface. Journal of Molecular Endocrinology 37: 317-26

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LIST OF ABBREVIATIONS

AADC	Aromatic amino acid decarboxylase
AAV	Adeno-associated virus
AAV-Cre	Cre-expressing adeno-associated virus
CA	Catecholamine
C	Caudal
CNS	Central nervous system
CPu	Caudate putamen
Cre	Cre recombinase
CRE-decoy	Decoy oligonucleotides containing a CREB DNA binding site
D	Dorsal
DA	Dopamine
DAT	Dopamine transporter
Dlk1	Delta-like 1
DMN-X	Dorsal motor nucleus X
DOPAC	3,4- dihydroxyphenylacetate
E	Embryonic stage
En	Engrailed
Fgf8	Fibroblast growth factor 8
Foxa	Forkhead/winged helix transcription factor
Gbx2	Gastrulation brain homeobox 2
GDNF	Glial cell line-derived neurotrophic factor
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
KA	Kainic acid
Klhl1	Kelch-like protein 1
L-DOPA	Dihydroxyphenylalanine
LB	Lewy body
Lmx1	LIM-homeobox transcription factor 1
Msx1	Msh homeobox transcription factor 1
NBRE-decoy	Decoy oligonucleotides containing a NR4A DNA binding site
Nor1	Neuron derived orphan receptor 1
NR	Nuclear receptor
Nur77	Nerve growth factor IB (NGFIB)
Nurr1	Nuclear receptor related 1
Otx2	orthodenticle homologue 2
P	Postnatal day
Pax	Paired-box transcription factor
PD	Parkinson’s disease
Pitx3	Pituitary homeobox transcription factor 3
Ptpru	Protein tyrosine phosphatase, receptor type, U
R	Rostral
RA	Retinoic Acid
RaldH1	Aldehydedehydrogenase 1a1 (Aldh1a1)
RRF	Retrorubral field
Shh	Sonic hedgehog
SNc	Substantia nigra pars compacta
TF	Transcription factor
TH	Tyrosine hydroxylase
V	Ventral
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area
Wnt1	Wingless-related MMTV integration site

INTRODUCTION

A single cell, the fertilized egg, gives rise to the adult human body consisting of approximately 10^{13} cells with hundreds of different cell types sharing the same genetic setup. This evokes questions as to how the different cell types are destined for their location and function in the adult body. These are fundamental questions of developmental biology and can be explained by a tightly regulated spatiotemporal pattern of intrinsic and extrinsic factors that coordinate critical events during development. The cellular identity is dependent on a unique combination of intrinsic transcription factors (TF's) that regulate a repertoire of genes.

The present thesis is focused on increasing our knowledge concerning transcription factor-mediated maintenance in neurons of the brain. This study is mainly focused on a type of brain neurons that utilize dopamine (DA) as neurotransmitter. In human, less than one million out of the 100 billion brain neurons use DA as neurotransmitter. Nevertheless, DA is critical for mediating voluntary movement, emotion, motivation and reward, and is implicated in the pathogenesis of psychological and neurological disorders such as schizophrenia, depression, drug addiction and Parkinson's disease (PD) (Dailly *et al.*, 2004; Maldonado, 2003; Nieoullon, 2002; Farrer, 2006; Klockgether, 2004).

The Dopamine System

DA was first recognized as a neurotransmitter in late 1950's, after years of having exclusively been thought of as a precursor of noradrenaline and adrenaline (Carlsson *et al.*, 1958). DA, noradrenaline and adrenaline are derived from the amino acid tyrosine and are collectively called catecholamines (CA).

Dopamine synthesis, storage and re-uptake

Tyrosine is taken up by amino acid transporters and once inside the cell it is converted to dihydroxyphenylalanine (L-DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH) (See Figure 1 for schematic illustration) (reviewed by (Elsworth and Roth, 1997). DA is then generated from L-DOPA by the enzyme aromatic amino acid decarboxylase (AADC). Because cytosolic DA can auto-oxidise and induce oxidative stress it is important that it is properly stored inside the neuron. DA is thus packed into

synaptic vesicles by the vesicular monoamine transporters (VMAT) and DA that is not immediately stored is degraded into 3,4- dihydroxyphenylacetate (DOPAC) through a two-step enzymatic reaction. DA-loaded vesicles are transported to the plasma membrane and upon an action potential the vesicles release their content into the synapse, where DA bind to receptors on the presynaptic and postsynaptic neuron. In the synapse, DA transmission is inactivated in synapses by re-uptaken of DA into neuron via the dopamine transporter (DAT) or by degradation of DA to homovanillic acid (HVA). These are important mechanisms in terminating DA neurotransmission and for maintaining synaptic homeostasis.

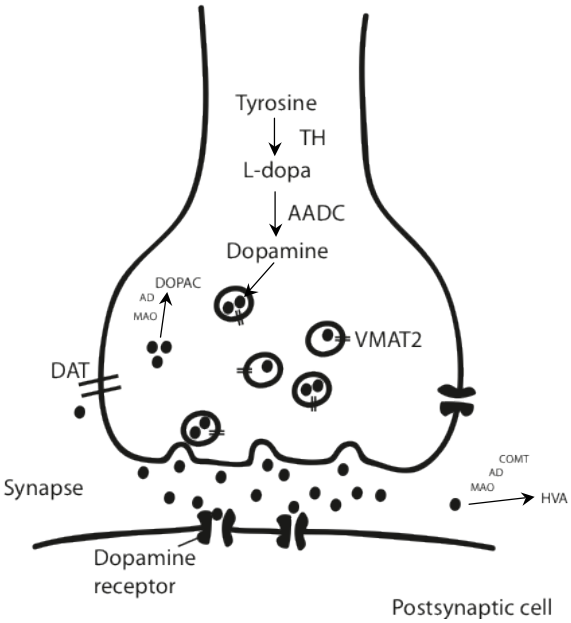

Figure 1. Schematic illustration of the DA cycle in the nerve terminal.
Abbreviations: MAO; monoamine oxidase, DOPAL; dihydroxyphenylacetaldehyde, AD; aldehyde dehydrogenase, COMT; catechol-*O*-methyl-transferase. See the test for further abbreviations. (Compliments of Stina Friling).

The distribution of DA neurons in the CNS

The first description of the distribution of CA-containing cells was done in the rat brain, and was later found to be applicable to mice and primates (Dahlström and Fuxe, 1964; Björklund and Lindvall, 1984). Seventeen groups of cells containing CA (referred to as A1-A17) are localized in the brainstem, olfactory bulb and retina (Figure 2). Nine of these utilize DA as a neurotransmitter and are found in the mesencephalon, also known as the ventral midbrain (A8-A10), in the diencephalon (A11-A15) and in the olfactory bulb (A16). However, the majority of the brain DA neurons (approximately 75%) are found in the midbrain (mDA), which corresponds to 20 000-30 000 in mice, 40 000-45 000 in rat and 400 000-600 000 cells in humans, where the DA system is increased in size and complexity (Bjorklund and Dunnett, 2007). In the present thesis, the summary of the mDA system will be based on our knowledge from rodents if nothing else is stated.

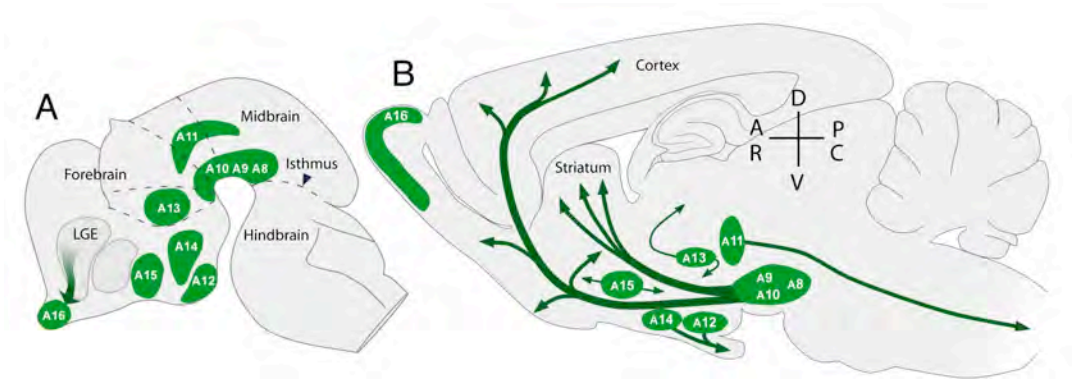

Figure 2. Schematic illustration of the distribution of DA neuron cell groups in the developing (**A**) and adult (**B**) rodent brain in a sagittal view. The DA neurons are localized in nine distinctive cell groups, found in the ventral midbrain (A8-A10), in the diencephalon (A11-A15) and in the olfactory bulb (A16). The majority of the brain DA neurons (approximately 75%) are found in the midbrain (mDA), which corresponds to 20 000-30 000 in mice, 40 000-45 000 in rat. Arrows in **B** illustrate the principal projections from the distinctive DA cell groups. The illustration is modified with permission from Prof. Björklund (Bjorklund and Dunnett, 2007). (Illustrator: Bengt Mattsson).

The Midbrain Dopamine Pathway

The ventral mDA neurons are divided into three anatomically distinct cell populations, referred to as the ventral tegmental area (VTA; A10), Substantia nigra pars compacta (SNc; A9) and the Retrorubral field (RRF; A8) (Dahlström and Fuxe, 1964). Their axonal projections form three functionally distinct DA pathways, the nigrostriatal (also known as mesostriatal), mesolimbic and mesocortical pathways, in which the two latter consists of an intermix of axonal projections originating from all three subpopulations (A8-A10) (Figure 3) (Bjorklund and Dunnett, 2007; Van den Heuvel and Pasterkamp, 2008). The DA neurons of the VTA and RRF are mostly projecting via the mesolimbic and mesocortical (mesocorticolimbic) pathway, which is important for the regulation of emotional and rewarding behaviours. A few scattered DA neurons of the SN are also a part of this system. A disorder of the mesocorticolimbic DA neurotransmission pathway is associated with the development of drug addiction, depression and schizophrenia (Dailly *et al.*, 2004; Maldonado, 2003; Nieoullon, 2002). The neurons of SNc are mainly innervating the dorsolateral striatum (the sensorimotor part of the caudate-putamen, CPu), forming the nigrostriatal pathway that is critical for the regulation of voluntary movements. These neurons are degenerated in patients with PD and have therefore been extensively studied (Klockgether, 2004; Farrer, 2006; Tanner, 1996).

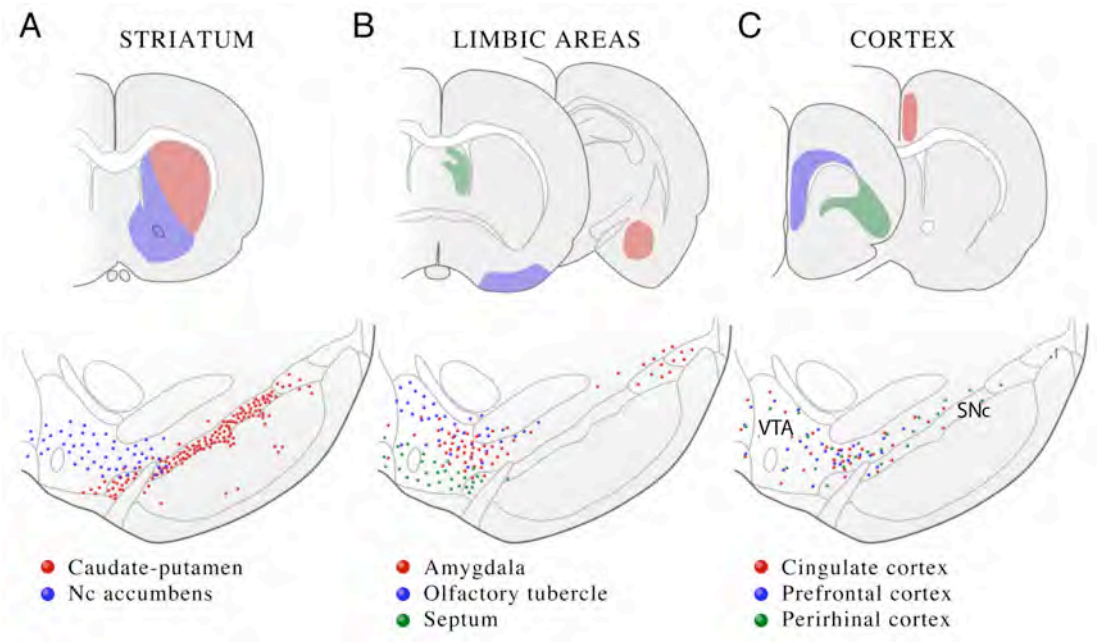

Figure 3. mDA neurons innervate striatal (A), limbic (B) and cortical (C) areas and their projections are partly intermixed. The dorsolateral striatum (red area in A) is innervated, probably exclusively, by the mDA neurons of SNc (red dots in A), which also comprise dorsally located neurons that project widely to both limbic and cortical regions (B and C). Adapted from (Bjorklund and Dunnett, 2007) with permission from Prof. Björklund. (Illustrator: Bengt Mattsson).

Parkinson's Disease

PD is the second most common neurodegenerative disorder after Alzheimer's disease. Approximately 0.1% of the world's population is suffering from this disease and the prevalence increases with age to reach about 1% of the population over 60 years (Tanner, 1996; Langston, 1998).

Symptoms and neuropathology

The PD symptoms were first described by James Parkinson for what he called a shaking palsy (Parkinson, 1817) and was later extended to three major motor symptoms including muscle rigidity, bradykinesia and resting tremor. In addition to the motor symptoms, many patients also develop cognitive impairments, anxiety, depression and autonomic dysfunctions (Owen *et al.*, 1992; Martignoni *et al.*, 1995; Aarsland *et al.*, 1999; Owen, 2004). The cause of this disease is, however, poorly understood. The neuropathological characteristics of PD are the loss of SNc DA neurons and the presence of intraneuronal inclusion called Lewy bodies (LB) or Lewy neurites if they are found in the axons or dendrites (Farrer, 2006; Klockgether, 2004). Lewy inclusions consists of abnormal aggregations of misfolded α-synuclein proteins (Schulz and

Falkenburger, 2004). The chronic and progressive loss of neurons in SNc result in a striatal DA depletion, and consequently in the movement difficulties displayed during disease. The symptoms arise when approximately 60% of the neurons are lost, which results in about 80% DA depletion in the putamen (Schulz and Falkenburger, 2004). It is notable that the degeneration of striatal nerve terminals precedes that of the cell bodies, suggesting that the terminals could be the primary destruction targets (Fearnley and Lees, 1991; Chung *et al.*, 2009). Pathology in other brainstem, subcortical and cortical regions of diverse neurons is also evident in the diseased brain (Braak *et al.*, 2003). For instance, serotonergic and noradrenergic system is affected in PD, and degeneration in these type of neurons is likely to account for the anxiety and depression displayed in the disease (Scatton *et al.*, 1983; Mann *et al.*, 1983; Remy *et al.*, 2005; Paulus and Jellinger, 1991). Furthermore, postmortem diseased brains reveal signs of inflammation within the SNc (McGeer *et al.*, 1988a; McGeer *et al.*, 1988b; McGeer and McGeer, 2008). Several inflammatory cues and activated microglia have been detected and are believed to take part in the pathogenesis of PD, reviewed in (Long-Smith *et al.*, 2009).

Treatment

At present, the only treatments available for PD are symptomatic ones. Administration of the DA precursor L-DOPA is the standard medical treatment. L-DOPA is converted to DA by the spared DA neurons, and also by serotonergic neurons. The serotonergic neurons have been shown to contain the enzyme AADC that converts L-DOPA to DA (Hokfelt *et al.*, 1973; Arai *et al.*, 1996; Arai *et al.*, 1995; Arai *et al.*, 1994). However, this therapy only restores the motor activity and as the disease progresses, a decreasing number of nerve terminals will be available to convert and store the DA. As a consequence the drug dose and frequency have to be increased, which in turn will cause severe side effects such as involuntary movements (dyskinesias) (Obeso *et al.*, 2000; Ahlskog and Muenter, 2001). In attempts to restore striatal DA transmission, several patients have been intrastriatally transplanted with fetal midbrain tissue but this is associated with ethical, economical and experimental difficulties and is therefore not considered as a alternative cure (Lindvall and Bjorklund, 2004). To generate a more accessible tissue, extensive work has been done to generate DA neurons by *in vitro* stem cell engineering. However, this requires a comprehensive understanding of the mDA neuron development, which is also a crucial first step in understanding the

disease progression and to reveal important concerns such as why the mDA neuron population in SNc is more vulnerable than in the VTA in the pathogenesis of PD.

Development of Midbrain Dopamine Neurons

Early patterning

The early patterning of the developing ventral midbrain domain is a highly complex and not yet fully understood process. The midbrain domain, in which DA neurons later appear, is first regionalized by the homeodomain TF's orthodenticle homologue 2 (Otx2) and gastrulation brain homeobox 2 (Gbx2), which by their reciprocal repression activity give rise to the midbrain-hindbrain boundary (MHB), also known as isthmus (Prakash and Wurst, 2004). Thereafter, several other TF's, such as Pax2/5, Lmx1b, Foxa1/2 and En1/2 as well as the secreted glycoprotein Wnt1 are expressed and are crucial for the second phase of regionalization (Smits *et al.*, 2006; Alavian *et al.*, 2008). (Please see the abbreviation list for the complete names of the factors mentioned in this subheading). A loss of any of these early patterning factors, with an exception for En2, results in failure to establishing the midbrain and hindbrain subdivisions or/and in generating mDA neurons (Acampora *et al.*, 1995; Wassarman *et al.*, 1997; McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Wurst *et al.*, 1994; Liu and Joyner, 2001; Bouchard *et al.*, 2000; Urbanek *et al.*, 1994; Schwarz *et al.*, 1997; Smidt *et al.*, 2000; Millen *et al.*, 1994). The midbrain domain is also regionalized along the rostral-caudal and dorsal-ventral axes by the two secreted factors fibroblast growth factor 8 (Fgf8) and Sonic hedgehog (Shh), respectively, which also induce the specification of mDA neuron precursors (see Figure 4 for a schematic illustration) (Hynes and Rosenthal, 1999). In summary, the midbrain regional patterning occurs through a spatiotemporal regulation of signalling cues and TF's expressed within this region, which specify the territory in which mDA neurons are born.

Specification of the identity

Shortly after the induction of the mDA neuron precursors, the expression of the two TF's Lmx1a and Msx1, both important for inducing neurogenesis and repressing alternative fates, are switched on in the proliferating cells (Andersson *et al.*, 2006). Intriguingly, Lmx1a is sufficient to drive Shh-ventralized stem cells into bona fide mDA neurons in culture (Andersson *et al.*, 2006; Friling *et al.*, 2009).

As soon as the cells become post-mitotic (embryonic stage (E) 10.5 in mouse), the orphan nuclear receptor Nurr1 (Nr4a2) is induced, and one day later the first signs of a DA neurotransmitter identity appear (Zetterstrom *et al.*, 1997). At the same time, other TF's, such as Pitx3, lmx1b and the Engrailed proteins, are expressed and are important for the normal development of mDA neurons as shown by gene targeting in mice (Zetterström *et al.*, 1997; Hwang *et al.*, 2003; Smidt *et al.*, 2000; Sgado *et al.*, 2006; Alavian *et al.*, 2008). The regulation of these TF's is poorly understood, as well as their downstream molecular mechanisms.

Interestingly, in *Pitx3*-deficient mice the loss of mDA neurons is restricted to the SNc, despite its ubiquitous expression in the entire mDA neuron domain (Hwang *et al.*, 2003; Nunes *et al.*, 2003; van den Munckhof *et al.*, 2003; Smidt *et al.*, 2004). A preferential failure of SNc neurons in inducing TH expression was also evident in these mutants (Maxwell *et al.*, 2005). This emphasizes that a difference in molecular mechanisms might exist between the distinct mDA subpopulations and that this could underlie the preferential neuronal vulnerability of SNc neurons that is also observed in PD (Chung *et al.*, 2005). One such signalling cascade might be mediated by the retinoic acid (RA), which is important in many neuronal mechanisms such as patterning, differentiation and axonal outgrowth (Jacobs *et al.*, 2007; Maden, 2007). The retinoic acid (RA)-synthesizing enzyme RaldH1, which is predominantly expressed in the mDA neurons of the SNc, is lost as a consequence of *Pitx3* deficiency (Jacobs *et al.*, 2007). The significance of this RaldHI loss on RA signalling in mDA neurons of Pitx3-deficient mice is yet unknown but a partial rescue of TH in SNc neurons was observed by maternal RA supplementation (Jacobs *et al.*, 2007).

The expression of several critical developmental TF's, such as Nurr1, Lmx1a/b, En1/2 and Pitx3, continues to be expressed in mDA neurons into adulthood, but very little is known about their functions in mature mDA neurons (Zetterström *et al.*, 1996b; Smidt *et al.*, 1997b; Smidt *et al.*, 2000; Simon *et al.*, 2004; Alberi *et al.*, 2004; Kittappa *et al.*, 2007). We have studied the consequences of *Nurr1* ablation in the late maturing and adult mDA neurons by using a strain of conditional Nurr1 knock-out mice and this is further discussed in this thesis, in the section "Results and Discussions" and in papers I and II.

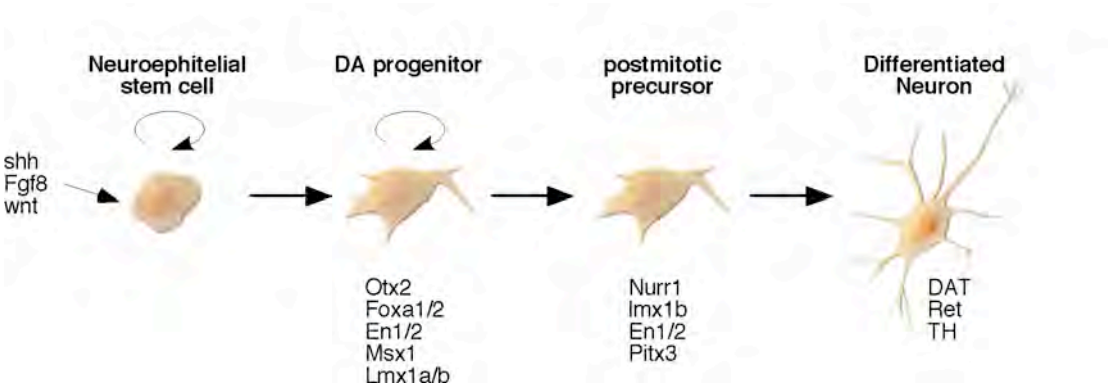

Figure 4. A Schematic overview of the development of mDA neurons, and factors that is important for their differentiation. (Cells illustrated by Mattias Karlén).

Nurr1

Nurr1 is critical for the neurotransmitter identity of mDA neurons by regulating the expression of genes important for DA synthesis, storage and re-uptake (Zetterström *et al.*, 1997; Smits *et al.*, 2003; Hermanson *et al.*, 2003). This regulation is restricted to the mDA neurons and is not critical for any other CA-cell population during development (Backman *et al.*, 1999; Le *et al.*, 1999a). As mentioned above, Nurr1 is expressed in the early post-mitotic mDA neurons and is essential for their development since the *Nurr1* null mice fail to induce a number of mDA markers (TH, DAT, VMAT2, and Ret) and are born deficient in mDA neurons (Zetterström *et al.*, 1997; Smits *et al.*, 2003; Wallen *et al.*, 2001; Perlmann and Wallen-Mackenzie, 2004). However, in the ventral midbrain of *Nurr1* null mice neurons are initially generated and show a normal induction of several markers including Pitx3, En1/2, RaldH1, FoxA2 and Lmx1b (Saucedo-Cardenas *et al.*, 1998; Wallén *et al.*, 1999; Smidt *et al.*, 2000; Smits *et al.*, 2003). During their late maturation, all mDA-specific markers are lost and the cells enter cell death by apoptosis (Saucedo-Cardenas *et al.*, 1998; Zetterström *et al.*, 1997; Castillo *et al.*, 1998; Wallén *et al.*, 1999). In addition, a deficiency of forebrain innervation was observed (Wallen *et al.*, 1999). The described role of Nurr1 in mDA neurons is currently too limited to explain the severe cellular phenotype observed in the *Nurr1* null mutants. Several potential target genes (TH, DAT, VMAT2, Ret and Dlk1) have been identified but a deficiency in any of these does not mediate the severe cellular consequences seen in *Nurr1*-ablated mDA neurons (Kim *et al.*, 2003; Sacchetti *et al.*, 1999; Hermanson *et al.*, 2003; Jacobs *et al.*, 2009; Wallén *et al.*, 1999). Thus, the downstream molecular mechanism for Nurr1 in DA cells remains to be unravelled. The role of two recently described putative target genes, *Klhl1* and

Ptpru, has not yet been described in the rodent mDA neurons but the latter has been implicated in the early patterning of the chick midbrain, before Nurr1's induction (Jacobs *et al.*, 2009; Badde *et al.*, 2005; Badde and Schulte, 2008).

The *Nurr1* null mice die within 24 hours after birth for reasons that are not yet fully understood (Zetterstrom *et al.*, 1997). However, the lethality is not due to Nurr1 functions in developing mDA neurons since DA deficiency caused by targeting *TH*-ablation specifically in DA neurons of mice is not lethal up to 3 weeks after birth. Moreover, these animals, in contrast to Nurr1 null mice, can be rescued by the DA precursor L-DOPA (Zhou and Palmiter, 1995). One suggested explanation for the early mortality of the *Nurr1* null mice is a severe respiratory defect due to *Nurr1*-deficiency in the brain stem regions controlling respiration, i.e. dorsal motor nucleus X (DMN-X), nucleus ambiguous, and solitary tract (Nsegbe *et al.*, 2004). Apart from a diminished expression of Ret in the DMN-X and a slight disorganization of associated axons, the brain stem looks grossly normal (Nsegbe *et al.*, 2004). Although, these brain stem regions appear histologically normal in the *Nurr1* null mice, their actual function may be dependent on the presence of Nurr1.

Nurr1 expression is mainly found within the CNS (Law *et al.*, 1992b; Zetterström *et al.*, 1996a; Saucedo-Cardenas and Conneely, 1996). In addition to the midbrain and brain stem, Nurr1 is also expressed in the cortex, hippocampus, thalamus and the spinal cord during development (Zetterström *et al.*, 1996a). Nurr1 expression pattern expand postnatally to also include the olfactory bulb, habenula, cerebellum and hypothalamus while it is diminished in the thalamus (Backman *et al.*, 1999; Zetterström *et al.*, 1996a). Outside the CNS, Nurr1 is found in the developing limb and in the adult testis, adrenal gland and thymus (Law *et al.*, 1992a; Saucedo-Cardenas *et al.*, 1998; Zetterström *et al.*, 1996a). The homologues Nor1 (Nr4a3) and Nur77 (Nr4a1) are also expressed in several of the above mentioned regions, such as cortex and hippocampus (Zetterström *et al.*, 1996a). However, neither Nor1 nor Nur77 is expressed in the ventral midbrain.

Nurr1 and its homologues – the NR4A subfamily

These factors share high structural homologies and together they constitute a highly conserved subfamily (referred to as NR4A) of orphan nuclear receptors. Nuclear receptors, as their name indicate, are abundant in the cell nucleus where they regulate genes by binding to DNA and recruiting coregulators (Aranda and Pascual, 2001).

Upon ligand binding, their ligand-binding domain (LBD) goes through a conformational transition that promotes the dissociation of corepressors and permits recruitment of coactivators, thereby mediating transactivation (Moras and Gronemeyer, 1998). The LBD of Nurr1 has been solved by X-ray crystallography and structural studies, which revealed a lack of a canonical binding site for co-activators and is further discussed in paper IV of this thesis (Wang *et al.*, 2003). It was also evident that Nurr1 lacks a ligand-binding cavity suggesting a ligand-independent function of Nurr1 (Wang *et al.*, 2003). Furthermore, comparison of the residues potentially critical for ligand binding of all three members indicates that Nur77 and Nor1 also lack the capacity to bind ligands. The question of how NR4A factors are regulated has remained uncertain but it has been suggested that they are regulated by post-translational modifications or by altered gene expression as they are encoded by immediate early genes that can rapidly be induced upon various stimuli (Maxwell and Muscat, 2006). The induction of NR4A proteins is rapid, strong and transient in the brain following focal brain injury, ischemia and seizure (Honkaniemi *et al.*, 1995; Honkaniemi *et al.*, 1997; Kim *et al.*, 2006; Honkaniemi and Sharp, 1999). Their expression can be induced by diverse stimulants, such as the glutamate analogue kainic acid (Honkaniemi and Sharp, 1999; Crispino *et al.*, 1998). This chemical compound binds to glutamate receptors (NMDA) leading to a dramatic calcium influx that causes intracellular excitotoxicity (Lothman and Collins, 1981). The significance of the acute induction of NR4A under such conditions has remained unknown until now and is further discussed in this thesis in manuscript III.

AIMS OF THIS THESIS

This thesis is focused on the transcriptional factor control mediated by Nurr1/Nr4a2, Nor1/Nr4a3 and Nurr77/Nr4a1, collectively referred to as NR4A. Unlike most nuclear receptors, NR4A proteins are the product of immediate early genes whose expression is acutely induced in response to various stimuli. The NR4A TF's are expressed predominantly in the CNS, being first detected during the early neuronal development, and persist throughout adulthood. Neurons in hippocampus and cortex express all three NR4A members whereas DA neurons of the ventral midbrain exclusively express Nurr1. Mice genetically targeted for *Nurr1* deletion show a selective and pronounced loss of developing mDA neurons, implicating Nurr1 as an important factor in the early differentiation of these neurons. As *Nurr1*-deleted mice are deficient in mDA neurons at birth and as Nurr1 continues to be broadly expressed in the CNS beyond the early specification into adulthood, it remains unclear if Nurr1 might also play a crucial role in maintaining mature neurons. The research presented in this thesis aimed to elucidate the role of the NR4A proteins in the brain, by targeting gene deletion in transgenic mice, and by loss/gain-of-function experiments in *in vitro* cultured neurons.

More specific aims:

1. To analyze the consequences of spatiotemporal *Nurr1*-deletion in the mDA neurons (paper I & II)

2. To characterize the function of stress-induced NR4A proteins in neurons (paper III)

3. To increase our insight into the mechanism by which Nurr1 mediates transcription (paper IV)

RESULTS AND DISCUSSION

Spatiotemporal ablation of Nurr1 in mDA neurons

Selective loss of mDA neurons in *Nurr1* null mice

Deletion of Nurr1 in mice resulted in a complete loss of mDA neurons followed by neonatal mortality (Zetterström *et al.*, 1997; Saucedo-Cardenas *et al.*, 1998). Twelve years later, our knowledge is still limited regarding the mechanistic basis of the severe cellular phenotype observed in *Nurr1* null mutants. Since Nurr1 expression persists into adulthood under normal conditions, Nurr1 may also play a crucial role in maintaining mature mDA neurons (Zetterstrom *et al.*, 1996). Consistent with this assumption, gene targeting in adult non-neuronal cell types has revealed that adult expression of the Hox-like homeoprotein Pdx1 is important for the maintenance of pancreatic cells (Holland *et al.*, 2002), Pax5 for B-lymphocytes (Cobaleda *et al.*, 2007) and Prox1 for lymphatic endothelial cells (Johnson *et al.*, 2008). To address the function of Nurr1 beyond the early differentiation, a mouse strain for conditional *Nurr1* gene ablation was generated by flanking the essential exon 3 of *Nurr1* with loxP sites ($Nurr1^{L2/L2}$). Conditional ablation was achieved by expressing Cre recombinase (Cre), which mediates site-specific DNA excision of residues flanked by loxP sites. We used different approaches to mediate Cre-recombination in the $Nurr1^{L2/L2}$ animals during late embryogenesis (E13.5), neonatal (Postnatal day (P) 0), young adult (P35) and adult (P75-P150) stages. An adult deletion of a TF that is critical during neuronal development had previously not been investigated in neurons.

Selective *Nurr1* ablation in late developing mDA neurons *(paper I)*

DAT-Cre-mediated Nurr1 ablation

To analyze the consequences of *Nurr1*-ablation at late stages of mDA neuron development, we crossed $Nurr1^{L2/L2}$ animals with mice expressing Cre under the DAT promoter. This breeding generated conditional *Nurr1* knock-outs ($Nurr1^{L2/L2}$;$DAT^{Cre/wt}$; referred to as $cNurr1^{DATCre}$). DAT expression is induced at E13.5 in mDA neurons, leaving a window of normal Nurr1induction and expression for three days (as Nurr1 is induced at E10.5) (Figure 5). By E15.5 the expression of Nurr1 was essentially lost in the mDA neurons. At this time-point during normal development, the mDA network is beginning to take shape as axons are innervating their target areas and pan-neuronal as

well as mDA-specific cell markers are detected. Importantly, the expression of DAT, Nurr1 and TH were not affected by the insertion of Cre under the DAT promoter as detected by immunohistochemistry on Nurr1$^{wt/wt}$;DAT$^{Cre/wt}$ brain sections.

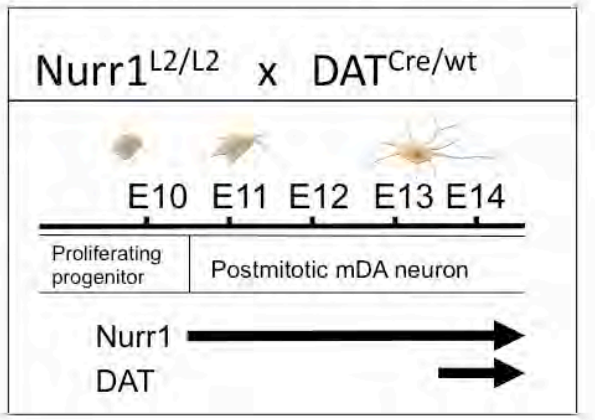

Figure 5. Experimental design. Nurr1$^{L2/L2}$ were crossed to DAT$^{Cre/wt}$ mice to generate late embryonic *Nurr1*-deficient animals. Nurr1 and DAT are induced at E10.5 and E13.5 respectively, leaving a window of three days of normal Nurr1 expression.

Premature death and abnormal behavior of cNurr1^{DATCre} mice

The cNurr1^{DATCre} animals were smaller than their wildtype littermates and were easily distinguished from the other genotypes already at P10. The mutants were born at the expected Mendelian frequency but did not survive beyond 3 weeks if no actions were taken. We managed to increase the survival frequency to approximately 50% by giving the mutants wet food as well as allowing the mutants to stay with their mothers after weaning. Nursing was further enhanced by reducing the litter size and/or separating the father to prevent further litters to be generated. Neither the decrease body weight nor the reduced viability of cNurr1^{DATCre} mice could be rescued by daily L-DOPA administration. In a spontaneous locomotor test the activity of adult cNurr1^{DATCre} animals was unaltered compared to their control littermates but they showed a dramatic decrease of rearing. Rearing is an exploratory act displayed by mice when placed in a novel environment. When rearing, the animals are standing upright on the hind-limbs while scanning the environment. In summery, late embryonic *Nurr1*-ablation in mDA neurons result in decreased body weight, rearing and viability of mice.

Reduced levels of mDA markers and DA within the brain of cNurr1^{DATCre} mice

To elucidate the fate of mDA neurons after *Nurr1*-ablation, we analyzed the expression of several mDA neuronal markers (TH, DAT, AADC, VMAT2, Pitx3 and Lmx1b) at the level of the ventral midbrain. All analyzed mDA markers were lost or diminished

within the SNc of cNurr1^{DATCre} animals already at E15.5, soon after the loss of Nurr1. The expression of DAT was completely lost, indicating that DAT might be a direct target of Nurr1, consistent with previous studies of DAT promoter (Sacchetti *et al.*, 1999). Later at birth, a deregulation of all the analyzed markers was also evident in the VTA of cNurr1^{DATCre} animals. Further expression analysis by TH immunostaining at postnatal stages revealed a progressive loss within the midbrain and striatal innervation area until adulthood in the mutants. However, a significant number of TH positive neurons remained in the VTA of adult cNurr1^{DATCre} animals. TH expression was completely undetectable in the early postnatal CPu, however, weak staining remained until adulthood in the NAc that is predominantly innervated by VTA neurons. The TH fiber-deficiency in CPu was accompanied by the appearance of ectopic TH-positive cells. These cells have previously been described to appear in rodent and primate DA-depleted models, reviewed in (Huot and Parent, 2007). Indeed we found decreased DA levels in cNurr1^{DATCre} mice by high performance liquid chromatography (HPLC) measurements. The DA levels in the CPu of cNurr1^{DATCre} animals were dramatically decreased to 14% of controls at birth and were almost completely lost by P60. DA levels in NAc were less severely decreased and showed 5% of normal levels at P60. In conclusion, a severe dysfunction of the mDA system is observed as a consequence of deleting Nurr1 during late embryogenesis.

Cellular deficiency as a consequence of late embryonic Nurr1-ablation

The expression pattern of the analyzed markers and the severe DA-depletion suggest a severe cellular phenotype as a consequence of Nurr1 ablation. In addition, SNc neurons expose a higher vulnerability to Nurr1 deletion than VTA neurons. In order to understand the cellular phenotype observed in the cNurr1^{DATCre} mutants, we performed a set of experiments. First, the striatal axons originating from midbrain neurons were traced by the fluorescent dye Fluorogold (FG). FG can be taken up by nerve terminals and retrogradely transported to the cell bodies (Schmued and Fallon, 1986). FG was injected unilaterally into dorsal CPu of adult mice, and was detected in the SNc of control animals. However, in the cNurr1^{DATCre} mutants no FG was detected in the mDA neurons indicating a disruption of the mDA pathway. Second, to address the possibility of neuronal loss, Nissel staining was performed on adjacent brain sections revealing a loss of the characteristic large and densely packed mDA neurons in cNurr1^{DATCre} animals. Taken together, these results suggest a rapid loss of mDA neurons within SNc in the cNurr1^{DATCre} mice, which later also progressed to affect the mDA neurons of

VTA. Our results reveal that Nurr1 is also critical during later stages of mDA maturation.

Selective Nurr1 ablation in postnatal mDA neurons *(paper I & II)*

Is Nurr1 also important for the adult integrity of mDA neurons?

Since Nurr1 has critical developmental functions and continues to be expressed postnatally, we wanted to understand its function in postnatal mDA neurons. Previous findings have implicated Nurr1 in maintenance of mDA neurons. Firstly, Nurr1 mutations and polymorphisms have been found in rare cases of PD (Xu *et al.*, 2002; Le *et al.*, 2003; Zheng *et al.*, 2003; Grimes *et al.*, 2006). However the link between Nurr1 and PD remains uncertain since other research groups have failed to show such an association (Wellenbrock *et al.*, 2003; Tan *et al.*, 2004; Hering *et al.*, 2004). Secondary, Nurr1 heterozygotes are more vulnerable to aging and dopaminotoxic compounds (Jiang *et al.*, 2005; Le *et al.*, 1999b; Pan *et al.*, 2008). However, the effects were relatively mild and as Nurr1 expression was decreased also during development in the Nurr1 heterozygotes, these studies did not establish if the effects were due to adult Nurr1 function or if they were a consequence of a developmental abnormality. Finally, Nurr1 expression is dramatically diminished within dystrophic SNc mDA neurons of PD postmortem brain tissue (Chu *et al.*, 2006; Le *et al.*, 2008). Although this finding demonstrates a Nurr1 deregulation in PD, it fails to establish if it is a cause or an effect of the pathology. Together these findings emphasize the importance of elucidating the potential function of Nurr1 in postnatal mDA neurons.

Virus-mediated Nurr1 ablation (paper I)

In a first attempt to ablate Nurr1 in adult mDA neurons, we used an adeno-associated virus type 5 that expressed Cre (AAV-Cre) under the neuron-specific synapsin promoter. Unilateral intracranial injections were conducted to induce *Nurr1* ablation on two-and-half to five months old $Nurr1^{L2/L2}$ animals (Figure 6). The AAV-Cre tested in reporter animals, that harbour a *LacZ* gene under a general promoter that contains a loxP-flanked stop codon (Soriano, 1999), showed a highly efficient recombination. Unfortunately, the AAV-Cre transduction frequency of the mDA neurons was unexpectedly low, with only a minority of the cells being transduced. However, in this first attempt we could demonstrate that Nurr1 is important for the maintained expression of genes important for the DA neurotransmission identity, as revealed by TH, DAT and VMAT2 immunostainings. For a more comprehensive study, we decided

to change our approach and therefore utilized a tamoxifen (Tam)-inducible Cre-expressing transgenic line (paper II).

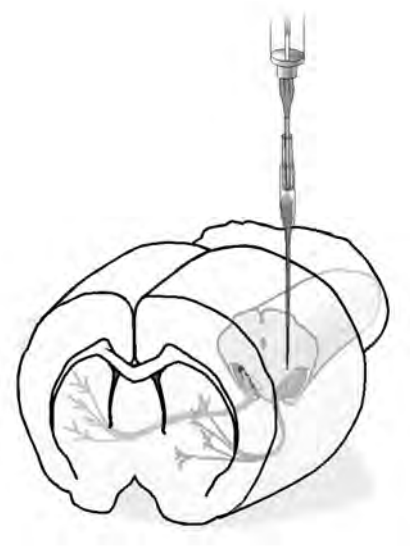

Figure 6. Experimental illustration demonstrating a unilateral intracranial injection just above the SNc of adult mice. In paper I, adult mice were injected with Cre-expressing virus to induce Nurr1 ablation in $Nurr1^{L2/L2}$ animals. (Illustrator: Bengt Mattsson, drawing was modified with permission).

Tam-inducible DAT-CreERT2 mediates complete Nurr1 ablation in mDA neurons

To further elucidate the fate of mDA neurons in the absence of Nurr1 we generated a mouse strain in which the *Nurr1* gene was genetically targeted in mDA neurons upon tamoxifen treatment. To achieve this, we used a transgenic mouse line carrying a Cre fused to a modified LBD of the estrogen receptor driven by the DAT promoter ($DAT^{CreERT2}$) (Engblom *et al.*, 2008). Breedings generated $Nurr1^{L2/L2}$;$DAT^{CreERT2/wt}$ and $Nurr1^{L2/L2}$;$DAT^{wt/wt}$ (control) animals that were administrated with 2mg Tam daily for five consecutive days beginning at P0 or P35. Upon treatment, a complete Nurr1 ablation was achieved in the mDA neurons of $Nurr1^{L2/L2}$;$DAT^{CreERT2/wt}$ animals, but not in the controls. The brain of the Tam-treated mice were histologically analyzed at one and four weeks post-treatment.

Spatiotemporal Nurr1 ablation

Our results show that Nurr1 continues to be important for the maintenance of the DA transmitter phenotype. The expression of TH, DAT, VMAT2 and AADC in mDA neurons were dysregulated as a consequence postnatal and adult *Nurr1*-ablation. TH expression was reduced significantly in both P0 and P35 *Nurr1*-ablated animals. But, a difference between the two ablated mutants was observed in the expression pattern of the remaining TH. TH expression was homogenously decreased throughout the entire

mDA network as a consequence of P35 *Nurr1*-deletion. However, TH was preferentially lost in SNc of the neonatal ablated animals and was only detected in VTA and in striatal regions innervated mainly by VTA neurons. Strikingly resembling the phenotype of late embryonic Nurr1 knock-outs (cNurr1^{DATCre}), described above and in paper I. Moreover, TH staining in the striatum of neonatal ablated mice revealed appearance of ectopic TH expressing cell bodies. Such ectopic cells did not appear in the young adult-ablated animals. (Ectopic TH expressing cells were also observed in the cNurr1^{DATCre} mutants, and have been described to appear in striatal DA-depleted primate and rodent models, as mentioned earlier in the text). In summary, our data revealed a time-course difference in the requirement of Nurr1 for the maintenance of TH expression.

Cellular maintenance in the absence of Nurr1

The preferential TH loss in the SNc of P0-ablated mice was not due to a cellular deficiency, since α-synuclein and AADC immunostained neurons in the SNc were detected. This finding highlights a difference between embryonic versus postnatal Nurr1-deficiency as a rapid cellular loss is detected during the former stage. The preferential loss of TH in SNc as a consequence of late embryonic Nurr1 deletion is accompanied by a rapid loss of SNc neurons. In the conventional Nurr1 null knockouts, TH fails to be induced in the ventral midbrain and the entire mDA domain goes through cell death before birth (Zetterström *et al.*, 1997; Saucedo-Cardenas *et al.*, 1998; Castillo *et al.*, 1998). Taken together, the temporal ablation of *Nurr1*, based on the observed TH expression pattern and the cellularity, indicate that time-course differences in the role of Nurr1 might exist between the mDA subpopulations. This hypothesis is further supported by the observed deregulation of Pitx3 and the transmembrane protein *delta-like 1 (Dlk1)* in the mDA neurons of neonatal but not young adult ablated animals. Moreover, the Pitx3-deficient mice show a preferential failure of SNc neurons in inducing TH expression followed by a prominent loss of mDA neurons within SNc, despite Pitx3 ubiquitous expression in mDA neurons during normal conditions (Smidt *et al.*, 1997a; Maxwell *et al.*, 2005; Smidt *et al.*, 2004; Hwang *et al.*, 2003; Nunes *et al.*, 2003).

Dramatic reduction of Ret in the absence of Nurr1

We next examined the expression of the receptor tyrosine kinase *Ret*, yet another mDA marker whose embryonic expression has previously been described to depend on Nurr1

(Wallen *et al.*, 2001). In an *in situ* hybridization assay we observed a dramatic decline in the levels of *Ret* in the postnatal and adult *Nurr1*-ablated mDA neurons. Previous findings have implicated Ret in the survival of mature mDA neurons as embryonic *Ret*-ablated mice show a progressive loss of SNc mDA neurons in aged animals (>12 months) (Kramer *et al.*, 2007; Jain *et al.*, 2006). Recently it was reported that an adult gene ablation of the glial cell line-derived neurotrophic factor (GDNF), that utilizes RET as its signalling receptor, resulted in a loss of mDA neurons at 7 months post-ablation (Pascual *et al.*, 2008). Thus, the maintenance of mature mDA neurons in *Nurr1*-ablated mDA neurons might not be long-lived as Ret was severely diminished as a consequence of *Nurr1*-ablation.

The role of the NR4A TF's in neuronal survival

NR4A-induction following neuronal stress *(paper III)*

As described earlier, the induction of the NR4A orphan nuclear receptors is rapid and robust in the brain following neuronal stress such as focal brain injury, ischemia and seizure (Honkaniemi *et al.*, 1997; Honkaniemi *et al.*, 1995; Honkaniemi and Sharp, 1999; Kim *et al.*, 2006). These are also associated with the activation of the TF cyclic AMP (cAMP) response element binding protein (CREB) (Lonze and Ginty, 2002). Previous loss-of-function studies in mice demonstrated that CREB is important in mediating neuroprotection. However, the mechanism by which CREB mediates such an activity is not well understood (Ao *et al.*, 2006; Lee *et al.*, 2009; Lonze *et al.*, 2002; Mantamadiotis *et al.*, 2002). In paper III we aimed at investigating if CREB is mediating neuroprotection through NR4A proteins. We used an *in vitro* system of cultured neurons with forebrain characteristics in which NR4A proteins and CREB were rapidly induced by stressful and pharmacological stimuli. Cultured neurons were exposed to ionomycin, glutamate and hydrogen peroxide to induce excitotoxic stress by the two former and oxidative stress by the later. In addition, the fate of hippocampal neurons exposed to neuronal stress was investigated in mice with null mutations in three out of six *Nr4a* alleles (*Nr4a2+/-*; *Nr4a3-/-* hereafter referred to as *NR4Amut*). Neuronal stress was induced by treating mice with kainic acid (KA), a glutamate agonist that is associated with excitotoxicity and oxidative stress within the hippocampus (Lothman and Collins, 1981). It is known that NR4A proteins are

robustly induced by KA stimulation (Honkaniemi and Sharp, 1999; Crispino *et al.*, 1998).

CREB-mediated NR4A induction upon excitotoxic and oxidative stress

Rolipram, a pharmacological type IV phosphodiesterase inhibitor, is known to activate CREB by inducing its phosphorylation (p-CREB) and to mediate neuroprotection upon various types of stress (Sasaki *et al.*, 2007; Zou and Crews, 2006). Our *in vitro* experiments demonstrated that Rolipram-enhanced resistance against stressful stimuli was hampered by titrating CREB away from its natural promoter targets (by using decoy oligonucleotides harbouring the CREB DNA responsive element; CRE-decoy). Rolipram-treated neurons showed an induction of the NR4A mRNA and protein levels. As well as a cellular colocalization of Nurr1 and p-CREB. Thus we generated an *in vitro* model in which the role of NR4A proteins in neurons can be studied in the absence of CREB transcriptional activity. Interestingly, NR4A-induction upon rolipram treatment was p-CREB-dependent as NR4A proteins failed to be fully induced when the normal CREB promoter-binding activity was prevented by CRE-decoy. Moreover, the stress mediated NR4A-induction by ionomycin and hydrogen peroxide were diminished in the presence of CRE-decoy. These together suggest that CREB mediates NR4A-induction after stressful stimuli. To address if *NR4A2* is a direct gene target of CREB, we analyzed the promoter region of NR4A2 and identified a putative CREB responsive binding site (CRE). To analyze the significance of this observation, cells were transfected with a reporter gene expressed from the wildtype or CRE mutated NR4A2 promoter. Reporter gene induction was detected after ionomycin or hydrogen peroxide treatment in the wildtype, but not in the mutated NR4A2 promoter. Furthermore, a chromatin immunoprecipitation (ChiP) confirmed p-CREB binding to this identified-binding site following stress-induction by ionomycin or hydrogen peroxide. In summary, NR4A proteins are directly and robustly induced by CREB in cultured neurons upon pharmacological or neuronal stress.

NR4A-mediated neuroprotection

We next investigated the survival of rolipram-pretreated cultured neurons after exposure to ionomycin, glutamate and hydrogen peroxide when NR4A proteins were titrated away from their natural promoter targets (by using decoy oligonucleotides containing a NR4A DNA binding site; referred to as NBRE-decoy). Significantly reduced survival was observed, however, the effect was not as severe as when CREB

activity was blocked by CRE-decoy. Our results suggest that the NR4A TF's are mediating a significant portion of the rolipram-induced neuroprotection. Moreover, a gain-of-function study in neurons transduced with lentivirus expressing each of the three NR4A proteins showed increased survival after neuronal toxicity induced by ionomycin, glutamate and hydrogen peroxide. To elucidate how NR4A proteins enhance resistance against neuronal stress we performed gene expression profiling by microarray analysis of neurons over-expressing NR4A2 or eGFP (control) (utilizing NR4A2- and eGFP-expressing lentivirus). The gene expression profiling revealed a significant upregulation of several genes (e.g. SOD1, C-FLAR and Adcyap1) that have previously been shown to mediate neuronal survival after stressful insults (Chan *et al.*, 1998; Krueger *et al.*, 2001; Vaudry *et al.*, 2002). We observed by quantitative real-time PCR (qRT-PCR) an upregulation of these genes when neurons were treated with the stable cAMP analogue 8CPT-cAMP (to mimic rolipram induction). Moreover, we further showed that the upregulation of these NR4A2-identified target genes upon 8CPT-cAMP was lowered when the transcriptional activity of NR4A proteins or p-CREB was prevented by NBRE-decoy and CRE-decoy, respectively.

NR4A-mediated neuroprotection in mouse hippocampus

To investigate if NR4A proteins mediate neuroprotection *in vivo* in cultured neurons, we administrated KA to animals. Importantly, KA-induced expression of NR4A in the mouse hippocampus has been demonstrated to be mediated by CREB (Lemberger *et al.*, 2008). Wildtype mice and mice deficient in three out of six *NR4A* alleles (*NRA2*+/-; *NRA3*-/- hereafter referred to as *NR4Amut*) were administrated with either saline or KA. qRT-PCR analysis of hippocampus tissue extracts revealed that several of the NR4A-identified target genes (Adcyap1, Prkaa2, Adm and C-FLAR) were induced by KA in the wildtypes. However, the levels were diminished in the *NR4Amut* animals. Histological analysis showed a significant decrease in neuron density within the hippocampal CA1 region of KA-treated *NR4Amut* mice. A less dramatic decrease in neuronal density was also evident in saline-treated *NR4Amut* mice as a consequence of NR4A3-deficiency as previously reported (Ponnio and Conneely, 2004). We further investigated the fate of the hippocampal neurons by staining for nitrosylated tyrosine species on oxidized proteins and with Fluoro-Jade to stain degenerating neurons. We found an increase of both markers within the CA3 region of the KA-treated *NR4Amut* mice. Taken together, our results demonstrate that animals deficient in three out of six

NR4A alleles are more vulnerable to neurodegeneration, presumably as a result of a decreased induction of a NR4A-dependent neuroprotective gene program.

Characterization of the Nurr1 ligand-binding domain

The ligand-binding domain (paper IV)

Upon ligand binding, nuclear receptors (NR) go through a conformational transition involving a reposition of the C-terminal α-helix (H) 12 of the canonical LBD (Moras and Gronemeyer, 1998). This conformational change exposes a well-defined surface called for activation function 2 (AF-2) that is recognized by coactivator proteins harbouring a LXXLL-amino acid motif (Nolte *et al.*, 1998; Shiau *et al.*, 1998). The coactivators are key components in mediating NR-dependent gene activation by inducing histone acetylation and other chromatin modifications (Xu *et al.*, 1999). Several findings indicate that Nurr1 mediates transcriptional activity by a mechanism distinct from other well-characterized NR's (Castro *et al.*, 1999). Firstly, none of the LXXLL-coactivators that have been shown to interact with other NR's, interact with Nurr1. Furthermore, the residues that have been described as essential for LXXLL-motif binding of coactivators are poorly conserved in Nurr1. Finally, the conserved residues of the AF2 surface of canonical NR's that have been shown to be important for their transactivation are not critical for the transactivation of Nurr1. Taken together, these findings indicate that Nurr1 might harbour an alternative LBD surface with which Nurr1 interacts with yet unknown coactivators. To increase our insight into the mechanism by which Nurr1 mediates transcription we further characterized the Nurr1 LBD by structural modelling.

Identification of a putative Nurr1 coregulator-binding site

To identify an alternative coactivator interaction surface we performed structural modeling of the surface of Nurr1 LBD. We found a non-polar hydrophobic groove suitable for protein-protein interaction at the opposite side of the AF2 core of the Nurr1 LBD. To asses the potential importance of this surface, site-directed mutagenesis was used to introduce mutations within this region. The mutations that were introduced resulted either in a deletion of a single amino acid or to a substitution to an alanine residue. Notably, Nurr1 LBD-mediated transactivation was essentially reduced or

abolished as a consequence of the mutations. In conclusion, these observations suggested that the identified hydrophobic surface might represent a coactivator-binding site that might recruit as yet unknown Nurr1 coregulators.

Nurr1 transcriptional activity is linked to decreased protein stability

Interestingly, one of the introduced mutations, a lysine substitution to an alanine residue (K577A) resulted in a dramatic increase of Nurr1 transcriptional activity. The increased activity could not be explained by higher levels of Nurr1 expression. In fact the expression levels were drastically decreased in cultured cells, as detected by western blot. We speculated that this was a consequence of increased protein turnover. To address that we treated Nurr1-K577A transfected cells with the proteosome inhibitor MG132 and we observed a strong increased expression level of Nurr1-K577A while the level was unaltered in wildtype control cells. Taken together, the decreased expression levels of the Nurr1-K577A derivate appears to be related with increased proteosome-dependent turnover. To test if the increased Nurr1 protein degradation was linked to its transactivation activity we introduced a second mutation in Nurr1-K577A. This second mutation resulted in a deletion of a residue that abolished Nurr1 transcriptional activity (K577A/delF598). Intriguingly, a strongly increased expression level of Nurr1- K577A/delF598 as compared to the single Nurr1- K577A mutant was detected in the transfected cell extracts. In conclusion, our results demonstrate an association between Nurr1 activity and protein turnover.

CONCLUDING REMARKS

The studies presented in this thesis clearly demonstrate that Nurr1 has an important function in the late developing and adult brain. Our results show that Nurr1 continues to be critical for the maintenance of mDA neurons during late embryogenetic development. In addition, we show that the maintenance of a DA transmitter phenotype is dependent on the continued activity of Nurr1 throughout life. Furthermore, we have successfully increased our insight into the mechanism by which Nurr1 and its homologues promote transcriptional activation and survival. Neuronal culture studies identified NR4A proteins as novel downstream neuroprotective-mediators of the stress-induced CREB. The stress-induced neuroprotective effect of Nr4a proteins was further confirmed in hippocampal neurons in adult *Nr4a* mutant mice (*Nr4a2+/-;Nr4a3-/-*). Finally, we identified a novel putative Nurr1 coregulator-binding site. Taken together, our data indicate that Nurr1, which is important during development, also play a crucial role in maintaining or inducing expression of several important genes in mature neurons. A deficiency of such a factor could lead to neurodegenerative diseases. Interestingly, several other TF's that are critical for neuronal differentiation continue to be expressed into adulthood.

ACKNOWLEDGEMENTS

Finally reaching the part where I can express my sincere gratitude to everyone who has contributed to this thesis by encouraging, helping and inspiring me over the years. I feel most fortune to have so many amazingly talented, generous and supportive colleagues and friends.

First and foremost I would like to express my deepest gratitude to my supervisor **Thomas Perlmann,** for giving me the opportunity to do this journey. Working in your lab has been most developing and enjoyable! Thank you for all the encouragement, support and inspiration!

Eliza, there is no words for how much I appreciate our friendship and collaboration. Thank you for teaching me all your special tricks. I will late forget our late nights at the microscope and your enthusiasm and passion for the project.

Gerard Benoit, my super-duper-visor. Thank you for all your supervision and support during my first couple of years.

Anders Björklund, Bengt Mattsson and **Ulla Jarl**. Thanks for kindly allowing me workspace in your lab, for the great scientific atmosphere and for making me feel like home. **Anders**, I have really liked to microscope with you. Thanks for sharing your deep knowledge in the dopamine research field. **Bengt & Ulla**, for sharing your expertise in the most generous way. **Bengt** for intracranial injections, behaviour, Canvas, confocal and for lunches. **Ulla**, for stainings, protocols and chats in the lab. Thanks for teaching me all these valuable techniques that I have been using over the last years.

Eva Hedlund, I am deeply grateful for everything you have done for me. For all the help, support, inspiration and encouragement. Thanks for your deep knowledge and for sharing your expertise. You are a truly generous and passionate scientist.

Maria Bergsland and **Nick Volakakis,** my fantastic friends! Thanks for your never-ending support, encouragements and friendship. It feels it was just yesterday we started at LICR.. sitting on “trappen”, sharing experiences and laughs. **Nick**, it has been great fun and inspiring to collaborate with you. **Maria,** you are amazing. Thanks for your words of wisdom. You probably understand me better then anyone else.

Idha Kurtsdotter, for all the chats about everything and nothing. For your amazing personality, skills and for all the support through rainy days.

Stina Friling, kompisen och stamcellsdrottningen. Thanks for all the help and support over the years. I miss you in the lab.

To colleagues in the lab; **Nigel**, **Magnus**, **Alex**, **Ariadna, Lia**, **Michal**, **CY, Jonas**, **Daniel**, **Cecilé, Holmberg, Soheilla** and **Susanne** for valuable discussions, help, support, friendship, parties, chats and lunches. The list is long and the memories are

numerous. Thank you all for contributing with great knowledge and atmosphere to the everyday worklife!

Thanks to all other **Ludwigians**, past and present, for creating an excellent scientific atmosphere.

The Ericson crew; **Johan**, **Qiaolin**, **Sanja**, **José**, **Tony**, **Mattias**, **Zhanna**, **Chris**, **Lizzy** and **Joanna** for valuable meetings, support and for your expertise in CNS and patterning.

Shirin, my curly-twin. Thanks for all the fun times and for “fredagsölen”.

Frisén’s angels, the gorgeous and fabulous C^{14}, ependymal and stem cell detectives, **Malin**, **Sofia**, **Hanna**, **Maria**, **Fanie** and **Kirsty**. For good friendship, nice chats, interesting science, parties, after-parties, pre-parties and aw.

Karin, Emma, Anne, Christine, och Tina. Tack för alla middagar, utflykter och vetenskapliga diskussioner. För att ni med er individuella forskning har berikat mig och bidragit till min utveckling som forskare. Ni strålar av genuinitet, lojalitet, intelligens och godhet. **Karin**, vår vänskap är något alldeles extra. Att försöka att sätta ord på det gör det inte rättvist. Tack för uni, London och PhD-tiden. Vi har varit oskiljaktiga.

Atosa och **Naya,** ni tillför så mycket glädje och skratt i mitt liv. Tack för att ni håller mig uppdaterad om den ”yttre” världen och för att ni alltid har förståelse med mina ”ovanliga” arbetstider samt mitt pendlande humör.

Khale Nahid, kära moster. För att du är en underbar person och vän. Jag är glad att ha dig så nära.

Neshat, **Martin** och **Amelie**, min alldeles egna underbara kusin med familj. Tack för att ni alltid ställer upp och för den stående middagsinbjudan. Ni berikar mitt liv!

Bahareh, Hamed och Kevin, stor-aja med familj. Tack för att ni alltid finns för mig, oavsett tid och rum. För att ni än idag låter mig vara lillasyster med allt vad det innebär. **Bahareh**, du är min bästa vän.

Poneh, lill-aja. Du är inte bara lillasyster utan även en bästa vän, reskamrat och buskamrat. Livet hade inte varit detsamma utan en lillasyster att styra upp massa hyss med.

Mami och babi, Tack för att ni förser mig med det enda som verkligen betyder något. För att dörren alltid står öppen för alla och för att ni alltid älskar ohämmat av hela era hjärtan. Ni är underbara!

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